35 U.S.C. § 112, second paragraph

Claims 1-12 were rejected for indefiniteness. As suggested by the Examiner, the claims were amended to relate back to the preamble. This rejection can now be withdrawn.

35 U.S.C. § 112, first paragraph

Claims 1-12 were rejected for lack of written description and lack of enablement.

With regard to written description, the Examiner stated:

> The claims broadly read on any sequence which could be considered an AFABP, from any mammalian organism, and any compound which could be considered an inhibitor of AFABP. The claims thus read on a broad scope of potential therapeutic agents which are not adequeatly describe by way of structure in the specification or in the art. (page 3, lines 10-13, of Paper No. 3)
>
> Both the specification and the art teach a correlation between AFABP reduced expression and a reduction in atherosclerosis. The specification as filed specifically teaches knock-out mice which do not express the AFABP gene. However, neither the specification nor the art teach design of inhibitors specific for AFABP. (page 4, lines 1-4, of Paper No. 3)
>
> Since the specification discloses only a knock-out of the AFABP gene in mice, and there is no teaching in either the specification nor the art for design of any inhibitor having a correlated function for use in any whole organism, Applicant would not have been in possession of the claimed inhibitors for use in the instantly claimed methods (paragraph spanning pages 4-5 of Paper No. 3)

Claims 1 and 12 have been amended to specify the amino acid sequence of AFABP (SEQ ID NO:4); claim 4 has been amended to require that the compound bind to a cis-acting regulatory sequence defined by SEQ ID NO:8; and claim 8 has been amended to require that an antisense sequence bind to an AFABP coding sequence (SEQ ID NO:2).

The design and structure of AFABP inhibitors is taught by the specification and is now defined by the amended claims. Applicants therefore request withdrawal of the rejection for lack of written description.

Enablement

Claims 1-12 were also rejected for lack of enablement. The Examiner's principal ground for rejection appears to be that the claimed invention falls within the art of antisense therapy. On pages 5-6 of the Office Action, the Examiner states:

> there is a high level of predictability know in the antisense art for therapeutic, *in vivo* (whole organism) applications. The factors considered to be barriers to successful delivery of antisense delivery to the organism are: (1) penetration of the plasma membrane of the target cells to reach the target site in the cytoplasm or nucleus, (2) withstanding enzymatic degradation, and (3) the ability to find and bind the target site and simultaneously avoid non-specific binding (see Branch). Depite synthesis of more resilient, nuclease resistant, oligonucleotide backbones and isolated successes with antisense therapy *in vivo*, the majority of designed antisense molecules still face the challenge of successful entry and localization to the intended target and further such that antisense and other effects can routinley be obtained. Flanagan teaches "oligonucleotides (in vivo) are not distributed and internalized equally among organs and tissues...Unfortunately, therapeutically important sites such as tumors contain very little oligonucleotide following intravenous injections in animals (page 51, column 2)"

The two publications upon which the Examiner relied in support of the enablement rejection date from 1998. Applicant submits that the claimed methods would not require undue experimentation because the state of the art of gene therapy has progressed significantly since the Branch and Flanagan articles were written. Specifically regarding Flanagan, Applicant notes that the difficulties raised by Flanagan, e.g., targetting to tumors, are not an issue in the present invention, because the target tissue (blood vessels) and target cells (macrophages) are easily acccessible by intravenous administration.

The fact that an invention falls within a particular art does not preclude patentability. Even in an "unpredictable" art, a number of factors must be weighed to determine whether the invention is patentable.

The factors to be analyzed in determining whether undue experimentation is required to practice the full scope of the claims are discussed in In re Wands.[1] The court in In re Wands set forth eight factors to be considered in determining whether undue experimentation would be required: (1) the state of the prior art, (2) the predictability or unpredictability of the art, (3) the breadth of the claims (4) the presence or absence of working examples, (5) the amount of direction or guidance presented, (6) the relative skill of those in the prior art, (7) the nature of the invention, and (8) the quantity of experimentation necessary.

Nature of the invention and state of the prior art

With regard to the nature of the invention and state of the art, the Examiner's position is that the nature of the art is antisense therapy and that the state of the art is that it is generally unpredictable.

Although the use of antisense is encompassed by the claims, the nature of the invention is inhibition of gene expression. Inhibition of gene expression can be accomplished using a variety of methods known in the art, e.g., antisense, ribozymes, organic compounds. The state of the art is that once a target gene is identified and its sequence determined, it is well within the skill of one practicing in the art of molecular medicine to reduce the expression of the target gene.

[1] In re Wands, 858 F.2d 731, 736-7 (Fed. Cir. 1988).

Applicants have made a significant contribution to the art by identifying a gene that is directly involved in the development of atherosclerosis, a progressive disease that affects millions of individuals.

Predictability or unpredictability of the art

The Examiner states that the art into which the invention falls is unpredictable, citing two publications from 1998 that report difficulties associated with delivering antisense oligonucleotides to target cells or tissues.

Applicants submit that the art of antisense may at one time have been considered an "unpredictable" art; however, the state of the art has changed such that antisense therapy is no longer considered unpredictable.

Antisense technology has been in development for nearly a decade. Successes with antisense therapy to specifically inhibit gene expression and reduce pathological symptoms in animals have been reported for several years. A number of antisense compositions targeting a variety of genes are currently in human clinical trials (http://www.isispharmaceuticals.com/pipeline.htm) for treatment of cancers, diabetes, and inflammatory and infectious diseases. At least one antisense nucleic acid composition has been approved by the Food & Drug Administration (FDA) for human therapy. For example, an antisense compound targeting viral DNA for the treatment of CMV retinitis in humans was approved by the FDA for marketing in the United States in 1998 (http://www.isispharmaceuticals.com/products/cmvpage.htm). Thus, the foundation for making and administering antisense compositions for treatment of disease is well established.

Breadth of the claims

As was discussed above, the claims have been amended to require inhibition of expression of AFABP, as defined by specific amino acid sequence (SEQ ID NO:4). The claims also require that the inhibitory compound bind to the nucleotide sequences of an AFABP coding sequence (SEQ ID NO:2). Moreover, the claims now require administration of compounds, which specifically bind to defined target sequences, e.g., SEQ ID NO:2 or 8). The scope of the claims is therefore commensurate with the disclosure provided in the specification of the application.

Presence or absence of working examples

Compliance with the enablement requirement of 35 U.S.C. 112, first paragraph, does not turn on whether an example is disclosed. An example may be "working" or "prophetic." A working example is based on work actually performed, while a prophetic example describes an embodiment of the invention based on predicted results rather than work actually conducted or results actually achieved.

In the present case, the specification contains one working example. The working example is a mouse, which is deficient in the expression of AFABP and apoE. The apoE model is an art recognized model for atherosclerosis. Mice with a null mutation in the genes for apoE (apoE -/-) or both apoE and AFABP (apoE -/-, AFABP -/-) were fed a Western diet (high fat) and compared. ApoE -/- mice maintained on the Western-type diet have cholesterol levels of greater than 1000. These mice also develop severe atherosclerosis, e.g., the carotid artery typically becomes 65-95% occluded. In contrast, in double mutant apoE -/-, AFABP -/- mice, few or no atherosclerotic lesions were detected. (page 6, lines 21-24, of the specification)

Related to the issue of the presence or absence of working examples is the issue of "correlation". "Correlation" refers to the relationship between *in vitro* or *in vivo* animal model assays and a disclosed or a claimed method of use. An *in vitro* or *in vivo* animal model example in the specification, constitutes a "working example" if that example "correlates" with a disclosed or claimed method invention.

The Examiner has acknowledged that the specification teaches a correlation between reduced AFABP expression and a reduction in atherosclerosis. (page 4, lines 1-4, of Paper No. 3). However, on page 6, lines 6-7, of Paper No. 3, the Examiner states: "Specifically, in vitro results with one antisense molecule are not predictive of in vivo (whole organism) success." The data described in the specificstion are *in vivo* (i.e., in a whole animal). The results in an art-recognized animal model for atherosclerosis were remarkable, i.e., "few or no atherosclerotic lesions were detected."

The working example described in the specification establishes that amended claims meet the statutory requirements for enablement.

Amount of direction or guidance presented

In addition to the working example, Applicants provide guidance regarding specific target sequences and specific nucleotides to be administered (pages 7, line 28, to page 9, line 7, of the specification). Contrary to the Examiner's assertion , the specification teaches design of inhibitors specific for AFABP. For example, the specification states that an inhibitory nucleic acid has a sequence at least 10 nucleotides in length (more preferably at least 20, 30, 40, 50 nucleotides in length) which is complementary at least a 10 nucleotide stretch of an AFABP cDNA (e.g., SEQ ID NO:2) (see page 8, lines 3-9, of the specification). Moreover, the

specification administration of antisense nucleic acid with a sequence complementary to that of SEQ ID NO:6 (see page 9, lines 6-7, of the specification).

For claims which require specific targetting of macrophage cells, the specification provides guidance regarding delivery of molecules by tethering them to macrophage-specific cell surface ligands (page 11, lines 1-13, of the specification). Methods for delivering nucleic acids are taught on page 14, lines 1-20, of the specification). With regard to identification of additional AFABP inhibitors, the specification provides extensive guidance as to testing compounds for their anbility to reduce AFABP expression or inhibit differentiation of macrophages into foam cells (page 14, line 21, to page 18, line 22, of the specification). Applicant therefore submits that the level of guidance presented in the specification is sufficient for one skilled in the art to carry out the claimed methods without undue experimentation.

Relative skill of those in the prior art

The skilled artisan in the relevant field is a molecular biologist or medical doctor. As was discussed above, the hurdles that remain in antisense technology (e.g., delivery issues raised by the Examiner) have largely been overcome. For example, success in antisense therapy to treat a vascular disease (hypertension) has been reported in animals and will proceed to clinical trials (Philips, I.M., 2002, Methods Enzymol 346:3-13). Applying the same protocols and principles to treating another vascular disease is well within the skill of those in the art. Although optimization will likely require some experimentation, it is not deemed to be undue for those skilled in the art of molecular medicine.

Quantity of Experimentation Necessary

In Wands, the Court stated,

> considerable amount of experimentation is permissible, if it is merely routine, or if the specification in question provides a reasonable amount of guidance with respect to the direction in which the experimentation should proceed.

Applying this criterion here, all of the techniques required to practice the claimed methods were described in the specification or were known to those skilled in the art as of the filing date.

Given the correlation between atherosclerosis and expression of AFABP that has been established in an art-recognized animal model for atherosclerosis, and the level of skill in the relevant art, Applicants submit that the undue experimentation would not be required of one skilled in the art to practice the claimed invention.

The issues raised by the Examiner fall into the category of routine experimentation, e.g., determination of formulation, dose, and mode of delivery, which is permitted by the statutory requirements for enablement. Applicant submits that the claimed methods would not require undue experimentation because the state of the art of gene therapy has progressed significantly since the Branch and Flanagan articles were written such that success is no longer out of reach. In fact, the Philips article to which Applicants refer above specifically indicates that antisense therapy is well suited for the treatment of vascular disease.

CONCLUSION

On the basis of the foregoing amendments and remarks, Applicant respectfully submits that the pending claims are in condition for allowance. If there are any questions regarding these amendments and remarks, the Examiner is encouraged to contact the undersigned at the telephone number provided below.

A petition for extension of time and a check in the amount of $920.00 is enclosed to cover the petition fee for a three month extension of time pursuant to 37 C.F.R. § 1.17(a)(3). The Commissioner is hereby authorized to charge any additional fees that may be due, or credit any overpayment of same, to Deposit Account No. 50-0311, Reference No. 21508-042.

Respectfully submitted,

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Dated: May 23, 2002

APPENDIX: MARKED-UP VERSION OF AMENDED CLAIMS

Amend claims 1, 2, 4, 8, and 12.

1. (amended) A method of inhibiting formation of an atherosclerotic lesion comprising administering to a mammal a compound that reduces expression of AFABP, wherein said AFABP comprises the amino acid sequence of SEQ ID NO:4 and wherein a reduction in AFABP expression inhibits formation of an atherosclerotic lesion.

2. (amended) A method of inhibiting formation of an atherosclerotic lesion in a mammal, comprising identifying a mammal in need of said inhibition, and introducing to said mammal a compound that reduces expression of AFABP, wherein said AFABP comprises the amino acid sequence of SEQ ID NO:4 and wherein a reduction in AFABP expression inhibits formation of an atherosclerotic lesion..

3. (reiterated) The method of claim 1, wherein said compound inhibits transcription of said AFABP.

4. (amended) The method of claim 1 wherein said compound binds to a cis-acting regulatory sequence of said AFABP, wherein said sequence comprises SEQ ID NO:8.

5. (reiterated) The method of claim 1, wherein said compound inhibits expression of said AFABP in macrophages but not in adipocytes.

6. (reiterated) The method of claim 1, wherein said compound inhibits expression of said AFABP in adipocytes but not in macrophages.

7. (reiterated) The method of claim 3, wherein said inhibitor is an antisense nucleic acid.

8. (amended) The method of claim 7, wherein said antisense nucleic acid molecule comprises at least 10 nucleotides the sequence of which is complementary to an mRNA encoding an AFABP polypeptide, said AFABP polypeptide being encoded by the nucleotide sequence of SEQ ID NO:2).

9. (reiterated) The method of claim 7, wherein said antisense nucleic acid is a DNA operatively linked to a macrophage-specific promoter, wherein transcription of said DNA yields nucleic acid product which is complementary to an mRNA encoding an AFABP polypeptide.

10. (reiterated) The method of claim 1, wherein said compound is introduced into an artery of said mammal.

11. (reiterated) The method of claim 1, wherein said compound is locally administered to a site of an atherosclerotic lesion in said mammal.

12. (amended) A method of inhibiting differentiation of a macrophage into a foam cell, comprising contacting said macrophage with an inhibitor of AFABP expression, wherein said AFABP comprises the amino acid sequence of SEQ ID NO:4 and wherein a reduction in AFABP expression inhibits differentiation of a macrophage into a foam cell.

Attachment A

Methods in Enzymology

Volume 346

Gene Therapy Methods

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[1] Gene Therapy for Hypertension: The Preclinical Data

By M. IAN PHILLIPS

Introduction

Although many excellent pharmacological agents are available commercially for the treatment of hypertension, the problems of cardiovascular disease related to hypertension continue to affect millions of people throughout the world. Hypertension is a multifactorial, multigenic disease, but the drugs aimed at controlling hypertension are aimed at relatively few targets. They target the renin–angiotensin system, β-adrenergic receptors or α-adrenergic receptors, and calcium channels. They should be very effective, but why is hypertension so widespread and morbid in our society and in the world?

Many of the drugs are expensive, and, therefore, unavailable to poor segments of all societies. Another problem is detection. Hypertension is undetected in about 40% of the population of the United States, according to the NHANES III Report.[1] Of that 40% in whom hypertension has been detected, about half receive treatment. The problem is further confounded because it is estimated that only 27% of those treated hypertensive patients fully comply with their treatment and have their hypertension controlled.[1] Clearly, there is a need for rethinking our approach to the treatment of hypertension. Detection could be increased by education. Nonpharmacological treatment, such as exercise, weight loss, and low salt diets, could provide inexpensive treatment, but it has proved very difficult to achieve compliance for these approaches. For treating hypertension on a world scale, we need something akin to an immunization against hypertension. Since hypertension is polygenic and not a single gene disease, except in very few cases[2] it cannot be immunized against. We need to develop ways that would improve hypertension control by providing longer lasting effects with a single dose and reducing side effects that lead to poor compliance. To do this, we began developing a somatic gene therapy approach in 1993[3,4] with the goal of producing prolonged control of hypertension. There have been two strategies taken: one by Chao and colleagues[5] to increase genes for vasodilation, and the other by Phillips and colleagues to decrease genes for vasoconstriction.[4] They represent the two sides to transferring

[1] N. M. Kaplan, "Clinical Hypertension" Williams and Wilkins, Baltimore, 1998.
[2] R. P. Lifton, *Science* **272**, 676 (1996).
[3] R. Gyurko, D. Wielbo, and M. I. Phillips, *Reg. Pept.* **49**, 167 (1993).
[4] M. I. Phillips, D. Wielbo, and R. Gyurko, *Kidney Intl.* **46**, 1554 (1994).
[5] J. Chao and L. Chao, *Immunopharmacology* **36**, 229 (1997).

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TABLE I
PRECLINICAL DATA ON GENE THERAPY FOR HYPERTENSION VASODILATOR GENES[a]

Target gene	Delivery	Model	Max Δ BP (mmHg)	Duration of effect	Reference
Human tissue	Adenovirus	Dahl-salt sensitive		4 weeks	Chao *et al.*, 1997[5]
Kallikren		5/6 renal mass	−37	5 weeks	Wolf *et al.*, 2000[42]
		SHR	−30	36 days	
	Adenovirus	SHR		5 weeks	Zhang *et al.*, 1999[43]
	Intramuscular	2K-1C	−26	24 days	
	Adenovirus iv	Doca-salt		23 days	Dobrzynski *et al.*, 1999[9]
Adrenomedullin	Adenovirus	Doca-salt	−41	9 days	Dobrzynski *et al.*, 2000[44]
	iv	SHR			Chao *et al.*, 1997[7]
	iv	Dahl-salt sensitive		4 weeks	Zhang *et al.*, 2000[45]
Atrial natriuretic peptide (ANP)	Adenovirus	Dahl-salt sensitive	−32	5 weeks	Lin *et al.*, 1997[6]
Nitric oxide	Plasmid	SHR	−21	5–6 weeks (1st injection) 10–12 weeks (2nd injection)	Lin *et al.*, 1997[8]

[a] *Abbreviations:* SHR, spontaneously hypertensive rat; AGT, angiotensinogen; AAV, adeno-associated virus; AT1-R, angiotensin type 1 receptor; ACE, angiotensin-converting enzyme; LNSV, retrovirus; AS-ODN, antisense oligodeoxynucleotide; CIH, cold-induced hypertension.

DNA into cells. One is the sense approach, i.e., the normal DNA sequence direction, and the other is the antisense approach, i.e., the opposite DNA sequence direction.

Sense to Vasodilation Genes

Chao *et al.* have an extensive series of studies on gene transfer to genes that act to increase vasodilator proteins (Table I). They have used genes such as kallikrein,[5] atrial natriuretic peptide,[6] adrenomedullin,[7] and endothelial nitric oxide synthase.[8] In different rat models of hypertension (SHR, Dahl salt-sensitive, Doca-salt) they showed that they could achieve blood pressure lowering effects for 3–12 weeks with the overexpression of these genes. The decrease in pressure resulting from

[6] K. F. Lin, J. Chao, and L. Chao. *Hum. Gene Ther.* 9, 1429 (1998).
[7] J. Chao, L. Jim, K. F. Lin, and L. Chao. *Hypertens. Res.* 20, 2692 (1997).
[8] K. F. Lin, L. Chao, and J. Chao. *Hypertension* 30, 307 (1997).

these vasodilator proteins ranged from −21 to −41 mmHg. The results of this group are consistent and impressive. Even though the effects were not very prolonged, there were reductions in end organ damage with these therapies.[9] However, the use of adenovirus limits the possibility of translating these strategies to humans. The use of plasmids, however, had very prolonged effects in their hands.

Antisense to Vasoconstrictor Genes

To counter overexpression of a gene as a critical factor contributing to hypertension, we introduced antisense somatic gene therapy. Antisense provides a highly specific, biological approach to produce attenuation of the sense DNA expression which produces too much protein: for example, angiotensin II (Ang II), which is responsible for increased vasoconstriction. Antisense gene therapy involves recombinant antisense DNA to express an antisense mRNA or antisense oligonucleotides to inhibit mRNA designed to specifically reduce an overexpressed protein that is critical to the disease. Since hypertension is a multigene disease, how can we decide on the candidate genes for gene therapy? We have ignored the difficulties of defining all the candidate genes by concentrating on the genes that have already been shown to be successful targets by experience with current drugs. These include beta receptors, angiotensin-converting enzyme (ACE), and angiotensin type 1 receptor (AT_1-R). Other targets follow logically, including angiotensinogen (AGT). Transfer of the antisense genes to somatic cells is achieved by an *in vivo* approach. It would be possible to try an *ex vivo* approach in which target cells are removed from the host, transduced *in vivo*, and then reimplanted as genetically modified cells. However, this strategy has no obvious applicability to hypertension, where the cause of the disease lies in the reaction of blood vessels but not in one specific tissue; even the heart, kidney, and brain are obviously very important in hypertension. The *in vivo* approach is challenging. One challenge is to provide sufficient antisense DNA, either alone or in a vector, to produce a sufficient concentration for uptake in a large number of cells. To do this we have developed two different strategies for hypertension gene therapy based on antisense with (a) antisense oligonucleotides (Table II) and (b) viral vectors to deliver antisense DNA (Table III).

Nonviral Delivery

Antisense Oligonucleotides

Antisense oligonucleotides are short lengths of synthetically made nucleotides (DNA) designed to hybridize with a specific sequence of mRNA. The hybridization has one or two effects: it stimulates RNase H or sterically inhibits the mRNA from translating its message in the read-through process at the ribosome, or it does both.

[9] E. Dobrzynski, H. Yoshida, J. Chao, and L. Chao, *Immunopharmacology* **44**, 57 (1999).

TABLE II
PRECLINICAL DATA ON GENE THERAPY FOR HYPERTENSION, VASOCONSTRICTOR GENES: ANTISENSE OLIGODEOXYNUCLEOTIDES[a]

Target gene	Delivery	Model	Max Δ BP (mmHg)	Duration of effect	Reference
AT1-R	AS-ODN icv	SHR	−30	Unknown	Gyurko *et al.*[3]
AGT	AS-ODN icv	SHR	−35	Unknown	Phillips *et al.*[4]
AT1-R	AS-ODN pvn microinjection	MRen2	−24	4 days	Li *et al.*[32]
TRH (Thyrotropin-releasing hormone)	AS-ODN intrathecal	SHR	−38	Unknown	Suzuki *et al.*[33]
AGE-2	AS-ODN portal vein	SHR	−20	6 Days	Morishita *et al.*[34]
Angiotensin-gene activating elements		SHR	−28	7 days	Nishii *et al.*[35]
Carboxypeptidase Y	AS-ODN HJV liposome	Doca-salt	−15	4 days	Hyashi *et al.*[36]
c-fos	AS-ODN microinection in RVLM	WKY SD	−16 −17	4–6 hours Unknown	Suzuki *et al.*[37]
CYP4A1	AS-ODN continuous infusion	SHR	16	Unknown	Wang *et al.*[39]
AGT	AS-ODN iv	SHR	−25	Unknown	Wielbo *et al.*[3]
AGT	AS-ODN hepatic vein HJV-liposome	SHR	−20	4 days	Tomita *et al.*[40]
AT1-R	AS-ODN icv	SHR	−30	7 days	Gyurko *et al.*[3]
AGT	AS-ODN with asialoglycoprotein iv	SHR	−30	7 days	Makino *et al.*[14]
AT1-R	AS-ODN iv	2K-1C acute	−30	>7 days	Galli *et al.*[41]
AT1-R	AS-ODN icv	2K-1C 6 months	−30	>5 days	Kagiyama *et al.*[17]
AT1-R	AS-ODN iv in liposomes	CIH	−38	Unknown	Peng *et al.*[17]
β1-AR	AS-ODN iv in liposomes	SHR	−35	30–40 days	Zhang *et al.*[10,11]

[a] *Abbreviations:* icv, intracerebroventricular; pvn, paraventricular nucleus; unknown, recovery of pressure not recorded.

Delivery of antisense oligodeoxynucleotides (AS-ODNs) can be carried out with direct injection of "naked DNA." We have found that direct injection is effective, but the efficiency of uptake is greatly increased by delivering the ODN in cationic liposomes provided the correct ratio has been calculated.[10]

[10] Y. C. Zhang, J. D. Bui, L. P. Shen, and M. I. Phillips, *Circulation* **101**, 682 (2000).

TABLE III
PRECLINICAL DATA ON GENE THERAPY FOR HYPERTENSION. VASOCONSTRICTOR GENES: VIRAL VECTOR DELIVERY OF ANTISENSE

Target gene	Delivery	Model	Max Δ BP mmHg	Duration of effect	Reference
AGT	AAV-based plasmid	SHR	−22.5	8 days	Tang *et al.*[21]
AT1-R	AAV ic	SHR adult	−40	9 weeks plus	Phillips[25]
AGT	AAV ic	SHR adult	−40	10 weeks plus	Phillips[25]
AT1-R	LNSV ic	SHR (neonates)	−40	90 days	Iyer *et al.*[27]
AT1-R	LNSV iv	SHR	−30	36 days	Katovich[38]
ACE	LNSV ic	SHR (neonates)	−15		Reaves *et al.*[31]
AGT	AAV ic	SHR (neonates)	−30	6 months	Kimura *et al.*[26]
AT1-R	AAV iv	Double transgenic mice (adult)	−40	6 months	Phillips *et al.*[29]

β_1-Adrenoceptor Antisense

Nonviral gene delivery, using cationic liposomes such as DOTAP and DOPE, have been successfully used by our group to deliver β_1-adrenoceptor antisense oligonucleotides (β_1-AR-AS-ODN) to act as novel beta blockers with prolonged effects.[10,11] By optimizing the liposome/ODN ratio and the incubation procedure, we are able to produce antihypertensive effects with β_1-AS-ODN for up to 33–40 days with a single dose.[11] The beauty of the β_1-AS-ODN is its specificity. The β_1-AS-ODN reduces β_1-adrenoceptors but does not affect β_2-adrenoceptors. Secondly, the β_1-AS-ODN does not cross the blood–brain barrier, and therefore, the novel β_1 blocker, based on antisense, will have no central nervous system side effects. The strongest uptake sites are in the heart and kidney where the β_1-adrenoceptors play a significant role. In the heart they control the force of contraction and this is reduced by the β_1-adrenoceptor. However, the heart rate is not affected by the β_1-AS-ODN.[1] This is in contrast to the effects of currently available beta blockers that have both β_1 and β_2 actions, and second, reduce heart rate as well as heart contractility. Therefore, the specificity offered by the ODN provides a more precise and accurate way of controlling the mechanisms contributing to high blood pressure without the side effects of bradycardia.[10] Furthermore, since the effect lasts for 30–40 days with a single injection, the antisense ODN is greatly superior to any of the currently available drugs, all of which have to be taken on a daily basis. Repeated injections intravenously (iv) at intervals of 3–4 weeks of β_1-AS-ODN produce prolonged control of high blood pressure without any toxic effects in the liver, blood, or organs.

[11] Y. C. Zhang, B. Kimura, L. Shen, and M. I. Phillips. *Hypertension* **35**, 219 (2000).

Angiotensinogen Antisense ODN

We have also established that angiotensinogen iAS-ODN is effective for antisense ODN for hypertension therapy. In human hypertension, the angiotensinogen gene has been shown to be linked and to play a role in the disease.[12] However, there is no currently available drug to inhibit angiotensinogen. We have designed antisense targeted to AGT mRNA and tested it *in vivo* and *in vitro*.[13] When given iv the angiotensinogen AS-ODN reduces blood pressure significantly when delivered with a liposome. These studies have been confirmed by others independently, showing that AGT-AS-ODN reduces blood pressure for up to 7 days with a single systemic dose.[14]

AT_1-R Antisense ODN

A similar story is true for the effects of AT_1-AS-ODN. This has been tested centrally with intracerebroventricular injections and with intravenous injections. It has been tested in spontaneously hypertensive rats (SHR)[15] and also in 2 kidney-1 clip animals[16] and environmentally induced hypertension.[17] In these three different models of hypertension, genetic, surgical, and environmental, the antisense produces a decrease in blood pressure within 24 hr of administration. The effect lasts for up to 7 days and there is no effect on heart rate.[15] The distribution of antisense is in blood vessels, kidney, liver, and heart.[17] The majority of uptake is in the kidney and liver.[17] A reduction in AT_1 receptors after treatment with the AT_1-AS-ODN reveals reductions in the protein in kidney, aorta, and liver.[17]

In summary, AS-ODNs have proved to be useful in demonstrating in the preclinical setting the power of AS-ODN to target specific genes and to reduce blood pressure for several days (or weeks) with a single administration. Laboratory data indicate that these effects are the result of rapid uptake of the antisense ODN into cells[18] where they migrate to the nucleus and inhibit the production of protein, mostly likely through translational inhibition of messenger RNA.[18,19] This could occur by the hybridization of ODN with specific mRNA, preventing the passage of the mRNA through the ribosome. Alternatively, DNA hybridization to RNA will in some tissues stimulate the production of RNase H for the specific sequence of mRNA bound to the ODN. RNase H destroys the RNA hybridized to DNA

[12] X. Jeunemaitre, F. Soubrier, Y. V. Kotelevetsev, R. P. Lifton, C. S. Williams, A. Charru, S. C. Hunt, P. N. Hopkins, R. R. Williams, and J. M. Lalouel, *Cell* **71**, 169 (1992).
[13] D. Wielbo, A. Simon, M. I. Phillips, and S. Toffolo, *Hypertension* **28**, 147.
[14] K. Makino, M. Sugano, S. Ohtsuka, and S. Sawada, *Hypertension* **31**, 1166 (1998).
[15] R. Gyurko, D. Tran, and M. I. Phillips, *Am. J. Hypertension* **10**, 56S (1997).
[16] S. Kaguyama, A. Varela, M. I. Phillips, and S. M. Galli, *Hypertension* (2001), in press.
[17] J.-F. Peng, B. Kimura, M. J. Fregly, and M. I. Phillips, *Hypertension* **31**, 1317 (1998).
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and thereby releases the oligonucleotide for further hybridization. This recycling action induced by RNase H may account for the long action of AS-ODNs.

Other useful features that make oligonucleotides attractive for hypertension therapy is that they can be produced relatively cheaply, rapidly, and in large quantities. The demand for oligonucleotides and primers has reduced the cost per base to a few cents. Second, they do not cross the blood–brain barrier and therefore, when given peripherally, will not have central effects.[10] Third, they are most effective when delivered in the right combination of ODN to cationic liposome.[10,11] Treatment of rats with liposome ODN complexes has not shown any toxicity in our experience.

Viral Vector Delivery

To produce very prolonged effects (i.e., several months) with a single injection, we use antisense DNA delivery by viral vector. Several viral vectors are available, but the adeno-associated virus (AAV) is both safe for use in humans and large enough to carry antisense genes with tissue-specific promoters.[20] The AAV is not to be confused with the adenovirus. Adenoviruses, although easy to use in lab animals, have caused a death in a human during trials and are not, in their present form, acceptable vectors. AAV is a parvovirus that does not replicate and does not induce inflammatory reactions. The AAV can be stripped of its rep and gag genes to carry up to 4.5 kb and deliver it to the nuclei of cells where it integrates in the genome.[21] When antisense DNA is used, the AAV allows the continuous production of an RNA that is in the antisense direction. This antisense RNA hybridizes to specific mRNA and inhibits translation. Therefore, we are developing antisense therapy using the AAV as a vector. To construct a viral vector requires the design and production of plasmids and gene packaging into the vector.

Delivery by Plasmids

Plasmids are effective vectors, but last for a shorter time than the viral vector because they do not allow integration into the genome. This is illustrated with the adeno-associated-based vector for angiotensinogen antisense cDNA.[22] A plasmid containing AAV terminal repeats was prepared with a cassette consisting of a CMV promoter, the rat AGT cDNA based on the sequence by Lynch *et al.*[23] The cDNA is oriented in the antisense direction. In addition, the cassette contains an internal ribosome entry site (IRES) and, as a marker, the green fluorescent protein gene

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(GFP).[24] At 48 hr after transfection into pAAV-AGT-AS, there was clear dominant expression of GFP in the H-4 cells. There was a significant reduction of AGT (120 ± 14 vs 230 ± 20 ng/mg protein, $p < 0.01$). Transgene expression detected by RT-PCR in the H-4 cells started at 2 hr and continued for at least 72 hr.

The plasmid was then tested *in vivo* by injecting the S and AS plasmids iv into SHR rats.[22] AGT-AS expression was positive in heart and lung at 3 days and 7 days. Expression in the kidney was absent or weak. When injected with 3 mg/kg plasmid, pAAV-AGT-AS produced a significant drop in blood pressure ($p < 0.01$) for 6–8 days in SHR. The drop in blood pressure correlated to a drop in plasma angiotensinogen levels that was significant at days 3 and 5 after injection. The decrease in blood pressure with injection of plasmid could be prolonged by injecting the plasmid with cationic liposome (DOTAP/DOPE).

Plasmids are useful for delivery of AS to produce an antihypertensive effect lasting about 1 week. They do not require the more complex packaging needed for recombinant AAV (rAAV).

Delivery by Recombinant AAV Vector

To produce long-term decreases in hypertension, we developed rAAV to deliver antisense to AT_1R in SHR.[20,25] The results showed that single intracardiac injection of rAAV-AT_1R-AS effectively reduced blood pressure by 30 mmHg for at least 5 weeks compared to controls.

To test whether an AAV delivery of an AT_1R antisense would inhibit development of hypertension, we injected 5-day-old SHRs. Hypertension in SHR develops between the eighth and tenth week after birth. Therefore, injecting in 5-day-old SHR allowed us to observe if the development of hypertension would be reduced. A single injection of AAV-AGT-AS in 5-day-old SHR significantly attenuated the full development[26] and level of hypertension for up to 6 months. In 3-week-old SHR rAAV-AT_1R-AS significantly reduced hypertension by about 30 mmHg for at least 5 weeks (the length of the study). However, unlike the reports of the effect of retrovirus delivery of an AT_1R-AS in 5-day-old SHR "curing" hypertension,[27] we did not find a complete inhibition of the rise in blood pressure.

In rAAV-AGT-AS treated SH rats, measures of plasma AGT levels showed a corresponding lack of increase in AGT in the AS treated groups, compared to the significant increase of AGT in the control animals.[26] Correlation of AGT versus blood pressure was significant ($p < 0.05$) in the control treated animals and not significant in the AS treated animals. This shows that angiotensinogen in the SHR

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is correlated with an increase in blood pressure. The AAV was expressed in kidney, heart, and liver throughout the time of the reduction in blood pressure. Thus, we concluded that early treatment with a single dose of rAAV-AGT-AS, given systemically, prevents the full development of hypertension in the adult SHR by a prolonged reduction in AGT levels. Similarly, the results with the rAAV-AT_1-AS showed a reduction in hypertension development correlated with a consistent reduction in AT_1 receptors in VSMC.[25] No toxicity was noted.[26] To prove the potential therapeutic value of rAAV, we have have used a mouse model of hypertension that clearly depends on an overactive renin–angiotensin system. In this model, which has human renin and human AGT trangenes, rAAV-AS-AT_1R reduced high blood pressure for up to 6 months with a single systemic injection.[28] This latest data with rAAV-AT_1R-AS confirms the results in adult SHR rats[20] and gives an even clearer picture that the AAV as vector has many advantages for hypertension therapy.

Other Vectors

Other vectors are being tested for hypertension gene therapy. As noted above, adenovirus vectors have been used with kallikrein gene insertion[5] and recently to deliver calcitonin gene-related peptide for hypoxia induced pulmonary hypertension in mice.[29] However, the adenovirus synthesizes proteins that trigger the immune system and cause inflammation, which limits its use in human therapy so far. Raizada and colleagues have worked with LNSV, a retrovirus, with antisense AT_1 receptor injected into newborn SHR to prevent the development of hypertension in the adults.[27,30,31] In a series of papers they report evidence that AT_1R-AS normalizes blood pressure and prevents organ damage. Retroviruses are appropriate only for dividing cells and therefore are not suitable for hypertension therapy in adults. The idea of injecting infants with AT_1R antisense on the chance

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they might have become hypertensive is questionable, but their studies offer a demonstration of antisense effectiveness. Retroviruses may be useful in treating cardiomyopathy, restenosis, and vascular remodeling, where cells are actively dividing, but retroviruses integrate randomly into the genome and the possibility of tumorigenesis is a high risk. Lentivirus vectors, which can infect dividing cells, are just beginning to be explored for therapeutic value. They offer large gene carrying capacity, are stable, and are easily produced. The disadvantage is the risk of uncontrolled infection and the potential for neoplastic changes. Other vectors, such as herpes simplex virus and Japan Sendai virus, are being tested as vectors, but all vectors are as yet only in limited use by certain laboratories.

Engineering Viruses

In addition to the choice of vectors, the control of transgene needs to be engineered and new promoters need to be explored before viral vectors can be used in humans.[30] The ideal promoter will be active for prolonged periods to maintain transgene expression and specific for a tissue cell type. The vector will need mechanisms to switch it on or off as required. This is being tested with the tetracycline transactivator system (tTA), by which a transgene can be activated in the presence (or absence) of tetracycline. Ultimately the promoters and transactivating factors will have to be so specific that the antisense can be turned on in a specific tissue when the need arises.

Conclusion

Both antisense oligonucleotides and antisense DNA delivered in a vector have advantages for gene therapy. Antisense ODNs can be used as drugs. They have an action that lasts for days or weeks, depending on how they are delivered. They are specific for a target protein and reduce overactive proteins, but because the inhibition is never total, they permit normal physiology. They are not toxic at therapeutic doses. ODNs can be produced in large quantities, relatively cheaply for humans. The challenge for antisense ODNs is to deliver them.

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The AAV vector with antisense DNA has very prolonged action (weeks/months) with a single dose, and is safe, nonpathogenic, and noninflammatory. The AAV is extremely stable. The challenge for clinical use is to increase production of large amounts at reasonable cost and to further engineer the control of the vector, as described above.

This brief review of some of the preclinical data shows that gene therapy for hypertension is possible.[30] The question is, Will these strategies be tested at the clinical level? The rAAV antisense strategy appears to be effective for reducing high blood pressure in different models of hypertension. Its development could provide a new generation of antihypertensive agents that would be administered in a single dose for prolonged effects lasting several months. Alternatively, antisense oligonucleotides are effective and highly specific. They could be used like long-acting drugs to provide sustained control of hypertension with infrequent administration. It seems that of the two strategies, the antisense oligonucleotides will be clinically acceptable first because of our familiarity with drug treatments. The viral vector approach will come much later, when all the basic science has been done to ensure that the patient is safe.

Summary

In spite of several drugs for the treatment of hypertension, there are many patients with poorly controlled high blood pressure. This is partly due to the fact that all available drugs are short-lasting (24 hr or less), have side effects, and are not highly specific. Gene therapy offers the possibility of producing longer-lasting effects with precise specificity from the genetic design. Preclinical studies on gene therapy for hypertension have taken two approaches. Chao *et al.*[5] have carried out extensive studies on gene transfer to increase vasodilator proteins. They have transferred kallikrein, atrial natriuretic peptide, adrenomedullin, and endothelin nitric oxide synthase into different rat models. Their results show that blood pressure can be lowered for 3–12 weeks with the expression of these genes. The antisense approach, which we began by targeting angiotensinogen and the angiotensin type 1 receptor, has now been tested independently by several different groups in multiple models of hypertension. Other genes targeted include the β_1-adrenoceptor, TRH, angiotensin gene activating elements, carboxypeptidase Y, c-*fos*, and CYP4A1. There have been two methods of delivery antisense; one is short oligodeoxynucleotides, and the other is full-length DNA in viral vectors. All the studies show a decrease in blood pressure lasting several days to weeks or months. Oligonucleotides are safe and nontoxic. The adeno-associated virus delivery antisense to AT_1 receptors is systemic and in adult rodents decreases hypertension for up to 6 months. We conclude that there is sufficient preclinical data to give serious consideration to Phase I trials for testing the antisense ODNs, first and later the AAV.